

Resolvins RvE1 and RvD1 attenuate inflammatory pain via central and peripheral actions

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Inflammatory pain, such as arthritis pain, is a growing health problem¹. Inflammatory pain is generally treated with opioids and cyclooxygenase (COX) inhibitors, but both are limited by side effects. Recently, resolvins, a unique family of lipid mediators, including RvE1 and RvD1 derived from omega-3 polyunsaturated fatty acid, have shown marked potency in treating disease conditions associated with inflammation^{2,3}. Here we report that peripheral (intraplantar) or spinal (intrathecal) administration of RvE1 or RvD1 in mice potently reduces inflammatory pain behaviors induced by intraplantar injection of formalin, carrageenan or complete Freund's adjuvant (CFA), without affecting basal pain perception. Intrathecal RvE1 injection also inhibits spontaneous pain and heat and mechanical hypersensitivity evoked by intrathecal capsaicin and tumor necrosis factor- α (TNF- α). RvE1 has anti-inflammatory activity by reducing neutrophil infiltration, paw edema and proinflammatory cytokine expression. RvE1 also abolishes transient receptor potential vanilloid subtype-1 (TRPV1)- and TNF- α -induced excitatory postsynaptic current increases and TNF- α -evoked *N*-methyl-D-aspartic acid (NMDA) receptor hyperactivity in spinal dorsal horn neurons via inhibition of the extracellular signal-regulated kinase (ERK) signaling pathway. Thus, we show a previously unknown role for resolvins in normalizing the spinal synaptic plasticity that has been implicated in generating pain hypersensitivity. Given the potency of resolvins and the well-known side effects of opioids and COX inhibitors, resolvins may represent new analgesics for treating inflammatory pain.

Resolution of acute inflammation, once thought to be a passive process, is now shown to involve active biochemical programs that enable inflamed tissues to return to homeostasis². The actions of proresolution mediators differ from currently used anti-inflammatory therapeutics. For example, inhibitors of COX and lipoxygenases disrupt resolution, because these enzymes are also required for the biosynthesis of proresolution mediators^{4–6}. Resolvins, such as RvD1 and RvE1, are biosynthesized from the omega-3 fatty acids docosahexaenoic acid and eicosapentaenoic acid, respectively, and show remarkable

potency in resolving inflammation-related diseases such as periodontal diseases, asthma and retinopathy^{2,3,7}. The peripheral and central mechanisms of inflammatory pain are not fully understood^{8–11}. Here we examined whether peripheral and central resolvins can attenuate inflammatory pain, and we further investigated how resolvins regulate the synaptic plasticity in spinal cord dorsal horn neurons that has been strongly implicated in the generation of persistent pain^{10,11}.

First, we examined the actions of RvE1 in an acute inflammatory pain model induced by intraplantar injection of formalin in CD1 mice. Formalin induces two phases of spontaneous pain behavior. The second phase is thought to be mediated via central mechanisms within the spinal cord^{12,13}. We delivered synthetic resolvins to the mouse spinal cord via the intrathecal route by lumbar puncture^{14,15}. Preemptive injection of RvE1 at very low doses, only 0.3 and 1.0 ng (that is, 1 and 3 pmol), decreased second-phase (10–45 min) but not first-phase pain behavior (0–10 min), suggesting a central mechanism of action for RvE1 (Fig. 1a,b). Notably, the effective dose range of RvE1 was much lower than that of either morphine or the COX-2 inhibitor NS-398 (Fig. 1c).

Next, we investigated whether RvE1's antinociceptive action is mediated by specific receptors. ChemR23, which is associated with the G protein subunit $G_{\alpha i}$, has been identified as RvE1's receptor^{16,17}. Spinal injection of the $G_{\alpha i}$ inhibitor pertussis toxin abrogated the action of RvE1 (Fig. 1d), suggesting a possible involvement of G protein-coupled receptors (GPCRs). Opioid receptors did not mediate the antinociceptive action of RvE1, as the opioid receptor antagonist naloxone reversed the effect of morphine but not that of RvE1 (Fig. 1d). Chemerin, a peptide agonist for ChemR23 (ref. 18), also attenuated formalin-induced second-phase pain in a dose-dependent manner (Fig. 1e). Notably, knockdown of ChemR23 with a specific siRNA abolished the antinociceptive actions of RvE1 (Supplementary Fig. 1). *In situ* hybridization revealed expression of ChemR23 mRNA in the dorsal root ganglion (DRG) and spinal cord (Fig. 1f). Double staining further showed expression of ChemR23 protein in DRG neurons that express TRPV1 (Fig. 1g and Supplementary Fig. 2) and in spinal cord cells that express the neuronal marker NeuN (Supplementary Fig. 3a–c). We also found ChemR23 in axons of DRG neurons (Fig. 1g) and primary afferent terminals in the

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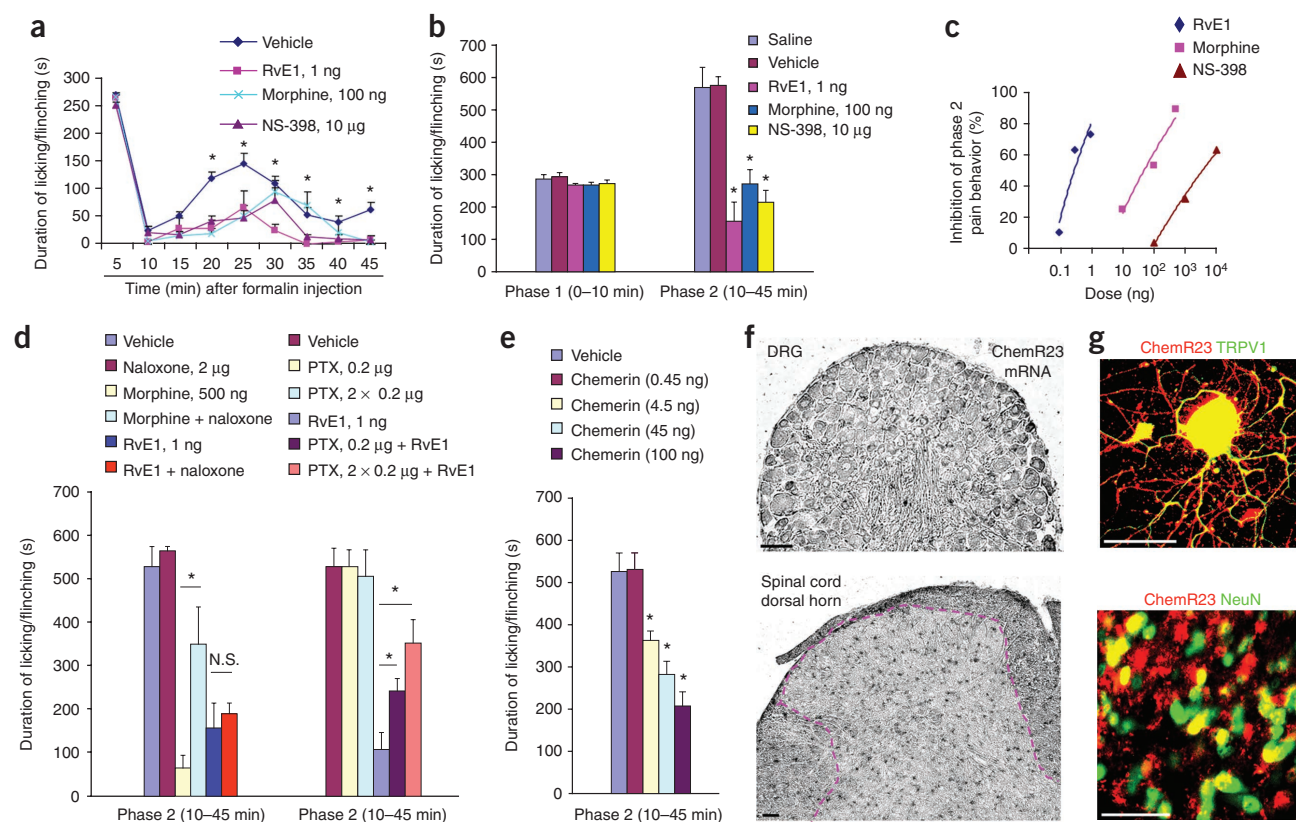


Figure 1 Preemptive spinal (intrathecal) administration of RvE1 reduces the second phase of formalin-induced inflammatory pain. **(a,b)** Reduction of the second phase of formalin-induced spontaneous pain by RvE1, morphine and the COX-2 inhibitor NS-398. **(a)** Time course. $*P < 0.05$ (vehicle versus RvE1). **(b)** First and second phase. $*P < 0.05$ versus vehicle, $n = 5-8$ mice. **(c)** Dose-response curve of percentage inhibition (versus vehicle control) of RvE1, morphine and NS-398 on the second phase of formalin-induced pain. $n = 5-8$ mice. **(d)** Reversal of RvE1-mediated inhibition of second phase pain by pertussis toxin (PTX) but not naloxone. NS, not significant. $*P < 0.05$, $n = 5-7$ mice. **(e)** Dose-dependent reduction of second-phase pain by the ChemR23 agonist chemerin. $*P < 0.05$ versus vehicle, $n = 6$ mice. **(f)** Expression of ChemR23 mRNA in the DRG and spinal cord dorsal horn, as revealed by *in situ* hybridization. Scale bars, 50 µm. **(g)** Colocalization of ChemR23 with TRPV1 in a cultured DRG neuron (top) and with NeuN in the superficial dorsal horn (bottom), as demonstrated by double immunostaining. Scale bars, 25 µm. All data are means \pm s.e.m.

spinal cord (**Supplementary Fig. 3d**). Therefore, RvE1 might attenuate inflammatory pain via ChemR23 expressed in DRG and spinal cord neurons.

Intraplantar injection of CFA elicits persistent inflammatory pain for weeks¹⁹. Intrathecal resolvins, given on day 3 after CFA injection, when heat hyperalgesia (reduction of paw withdrawal latency) is fully developed (**Fig. 2a**), attenuated hyperalgesia in a dose-dependent manner (**Fig. 2b,c**). Notably, 10 ng of RvE1 produced an ~75% reduction in hyperalgesia 15 min after administration (**Fig. 2b**). A meta-analysis previously showed that dietary omega-3 fatty acids alleviate inflammatory joint pain in patients²⁰. The omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid, the respective precursors of RvE1 and RvD1, also reduced the severity of CFA-evoked heat hyperalgesia (**Fig. 2b**). But the effective doses of eicosapentaenoic acid and docosahexaenoic acid required were 10,000 times higher than that of RvE1 (**Fig. 2b**). For direct comparison, 10 ng RvE1 was more potent than 10 µg of the COX-2 inhibitor NS-398 (**Fig. 2c**). Notably, a stable analog, 19-(p-fluorophenoxy)-RvE1 (19-pf-RvE1), designed to resist rapid local metabolic inactivation of RvE1 (ref. 21), reduced hyperalgesia for 6 h (**Fig. 2c**). By contrast, a further metabolic product of RvE1, 18-oxo-RvE1 (ref. 21), was essentially inactive (**Fig. 2c**). Although RvE1 potentially reduced inflammatory pain,

it did not alter baseline sensory thresholds in naive mice (**Fig. 2d**). These findings suggest that resolvins have a unique role in the normalization of inflammatory pain.

We further investigated the peripheral role of resolvins in carrageenan-elicited pain and inflammation. Intraplantar pretreatment with RvD1 and RvE1 substantially attenuated carrageenan-induced heat hyperalgesia (**Fig. 2e**). As expected, RvE1 had marked anti-inflammatory effects, reducing carrageenan-induced edema, neutrophil infiltration and expression of proinflammatory cytokines (for example, TNF- α , interleukin-1 β (IL-1 β) and IL-6) and chemokines (for example, monocyte chemoattractant protein-1 and macrophage inflammatory protein-1 α) in inflamed hindpaws (**Fig. 2f-h** and **Supplementary Fig. 4**). Intraplantar RvE1 also rapidly attenuated formalin-induced acute pain (**Supplementary Fig. 5a**).

To determine the potential mechanisms by which resolvins attenuate inflammatory pain, we examined the impact of RvE1 on TNF- α signaling, because TNF- α is a key contributor to the genesis of inflammation and pain via both peripheral^{22,23} and central²⁴ mechanisms. Indeed double knockout mice lacking both *Tnfr1* and *Tnfr2* (*Tnfrsf1a*^{-/-}; *Tnfrsf1b*^{-/-}) showed a marked attenuation in CFA-elicited heat hyperalgesia and formalin-elicited second-phase pain (**Fig. 3a**). Intrathecal injection of TNF- α also

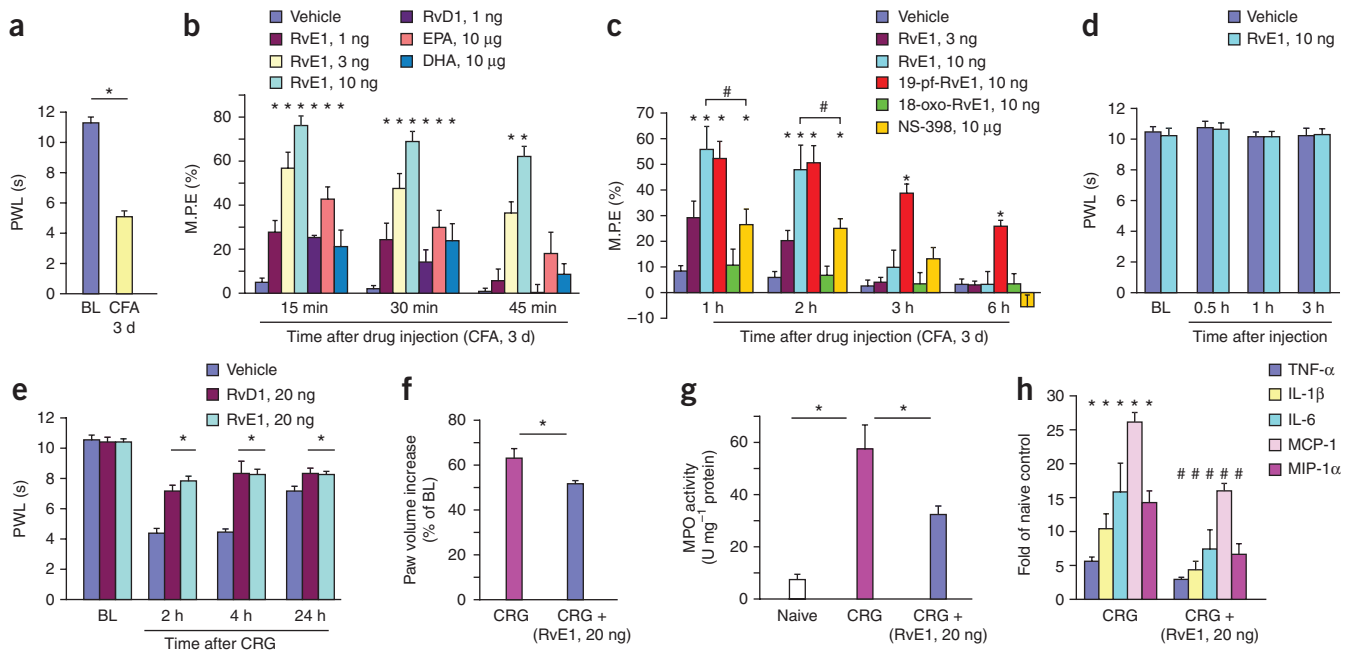


Figure 2 Central and peripheral actions of resolvins on persistent inflammatory pain and inflammation. (**a–d**) Effects of intrathecal administration of resolvins on day 3 after CFA treatment on CFA-evoked heat hyperalgesia. (**a**) Development of heat hyperalgesia 3 d after CFA injection. (**b**) Acute effects (15–45 min) of RvE1, RvD1, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). (**c**) Persistent effects (1–6 h) of RvE1, NS-398 and 19-pf-RvE1. (**d**) Lack of effect of RvE1 on basal pain thresholds in naive mice. PWL, paw withdrawal latency; MPE, maximum possible antihyperalgesic effect. * $P < 0.05$ versus baseline (BL, **a**) or vehicle (**b–d**); # $P < 0.05$, $n = 4–7$ mice. (**e–h**) Quantification of carrageenan (CRG)-elicited heat hyperalgesia (**e**), paw edema (**f**), neutrophil infiltration (**g**) and expression of proinflammatory cytokines and chemokines (**h**) in the inflamed paw after intraplantar pretreatment of resolvins. Edema, neutrophil infiltration and cytokine expression at the protein level were examined by paw volume (**f**), myeloperoxidase (MPO) activity (**g**) and cytokine array (**h**), respectively, at 4 h (**f,g**) or 2 h (**h**) after carrageenan injection. * $P < 0.05$ versus vehicle (**e,f**) or naive (**g,h**), # $P < 0.05$, versus CRG (**h**), $n = 3–6$ mice. All data are means \pm s.e.m.

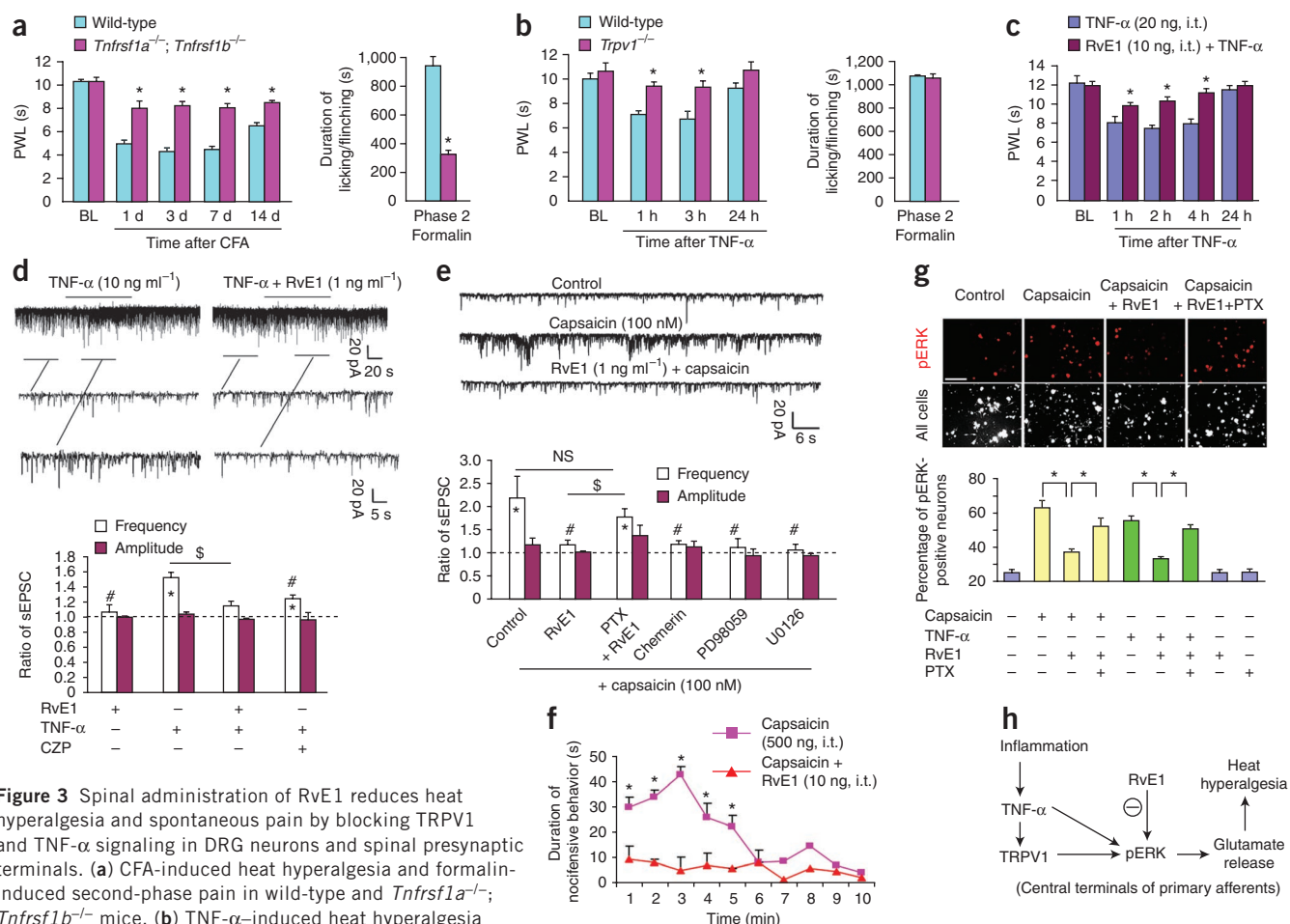
evoked marked heat hyperalgesia, which was abrogated in mice lacking *Trpv1*, a crucial gene for generating heat hyperalgesia²⁵. In contrast, formalin-induced second-phase spontaneous pain was unaltered in *Trpv1*^{-/-} mice (**Fig. 3b**). Spinal administration of RvE1 substantially reduced TNF- α -induced heat hyperalgesia (**Fig. 3c**). Hence, RvE1 can alleviate both TRPV1-dependent and TRPV-independent inflammatory pain symptoms.

To determine whether resolvins modulate spinal cord synaptic plasticity, which is thought to underlie persistent inflammatory pain^{10,11}, we used a patch-clamp technique to record spontaneous excitatory postsynaptic currents (sEPSCs) in lamina II neurons *ex vivo* in isolated spinal cord slices from mice. Perfusion of spinal cord slices with TNF- α induced an increase in the frequency but not amplitude of sEPSCs (**Fig. 3d**), suggesting a presynaptic effect of TNF- α achieved by increasing glutamate release from axonal terminals²⁴. Notably, RvE1 alone did not alter basal synaptic transmission but did block the TNF- α -induced increase in sEPSC frequency (**Fig. 3d**). The TRPV1 antagonist capsaizine also reduced this increase in sEPSC frequency by TNF- α (**Fig. 3d**), in parallel with earlier results that TNF- α increased TRPV1 activity in DRG neurons^{26,27}. Direct activation of TRPV1 by capsaicin (100 nM) elicited a twofold increase in sEPSC frequency that was completely blocked by RvE1 (**Fig. 3e**). Like RvE1, chemerin also abolished this frequency increase by capsaicin in a pertussis toxin-dependent manner, suggesting the involvement of ChemR23 (**Fig. 3e** and **Supplementary Fig. 6**). Notably, intrathecal capsaicin elicited acute spontaneous pain for <10 min, and intrathecal RvE1 prevented this induction of acute pain (**Fig. 3f**). In parallel, peripheral RvE1 reduced intraplantar capsaicin-induced acute pain (**Supplementary Fig. 5b**). These results further

establish that RvE1 attenuates inflammatory pain by blocking TRPV1 and TNF- α signaling, presumably at presynaptic sites.

Next, we investigated whether resolvins modulate synaptic plasticity via the ERK signaling pathway, because previous studies have reported that ChemR23 regulates ERK signaling in non-neuronal cells¹⁸, ERK activation in DRG neurons increases TRPV1 activity²⁸ and ERK modulates neurotransmitter release via phosphorylation of synapsin I²⁹. We inhibited the ERK pathway with the mitogen-activated protein kinase (MEK) inhibitors U0126 and PD98059 and found that both blocked the capsaicin-induced sEPSC increase, indicating a role for ERK in regulating presynaptic glutamate release in the spinal cord (**Fig. 3e**). In dissociated DRG neurons, both TNF- α and capsaicin elicited increases in phosphorylation of ERK (pERK), and RvE1 abolished these increases (**Fig. 3g**). Thus, RvE1 might attenuate inflammatory pain by blocking ERK-mediated glutamate release in presynaptic terminals in response to TNF- α stimulation and TRPV1 activation (**Fig. 3h**).

Apart from heat hyperalgesia, CFA and intrathecal TNF- α produced another cardinal feature of inflammatory pain, mechanical allodynia (a reduction in paw withdrawal threshold). Intrathecal RvE1 also attenuated mechanical allodynia induced by TNF- α or CFA (**Fig. 4a** and **Supplementary Fig. 7a,b**). Of note, TNF- α -induced mechanical allodynia was TRPV1 independent (**Fig. 4b**). To define potential mechanisms by which RvE1 attenuates mechanical allodynia, we examined the activation of glutamate NMDA receptors (NMDARs) in dorsal horn neurons, which results in hyperactivity of these neurons (central sensitization) and, subsequently, mechanical allodynia^{10,19}.



We measured the activity of NMDARs by recording NMDA-induced currents in dorsal horn neurons. TNF- α significantly potentiated NMDA currents, and RvE1 reduced this potentiation (Fig. 4c). We further assessed whether RvE1 inhibits NMDAR activation via the ERK pathway, as ERK phosphorylation in dorsal horn neurons serves as a marker for central sensitization^{13,30}. Perfusion of spinal slices with TNF- α induced robust ERK phosphorylation primarily in superficial dorsal horn neurons, and RvE1 treatment reduced the phosphorylation (Fig. 4d). We found that ERK mediates central sensitization via activation of NMDARs in postsynaptic dorsal horn neurons, because the MEK inhibitors PD98059 and U0126, but not capsazepine, blocked TNF- α -induced NMDAR activation (Fig. 4e). Thus, we postulate that RvE1 also attenuates inflammatory pain by inhibiting ERK-mediated NMDAR activation in postsynaptic dorsal horn neurons (Fig. 4f).

In addition to dampening behavioral hypersensitivity in inflammatory pain conditions, resolvins also reduced mechanical or heat hyperalgesia in other persistent pain states, including incision-induced

postoperative pain (Supplementary Fig. 7c) and nerve injury-induced neuropathic pain (Supplementary Fig. 7d).

In summary, these results show that resolvins, at very low doses (0.3–20 ng), effectively reduce inflammatory pain symptoms in several mouse models, through both peripheral and central actions. Biosynthesized during resolution of acute inflammation, resolvins are known to act on immune cells via anti-inflammatory mechanisms (for example, reducing polymorphonuclear leukocyte infiltration and tissue injury) and proresolving actions (for example, increasing phagocytic activity of macrophages)². As expected, peripheral administration of RvE1 reduced carrageenan-elicited expression of proinflammatory cytokines, neutrophil infiltration and paw edema. Because proinflammatory cytokines such as TNF- α and IL-1 β are indispensable for the pathogenesis of inflammatory pain (Fig. 3a,b)^{24,31}, the antinociceptive effects of resolvins could be attributable to its anti-inflammatory role. Also, it is noteworthy that we show here a previously undescribed mechanism in pain resolution where RvE1 rapidly, within minutes, decreases inflammatory pain by modulating

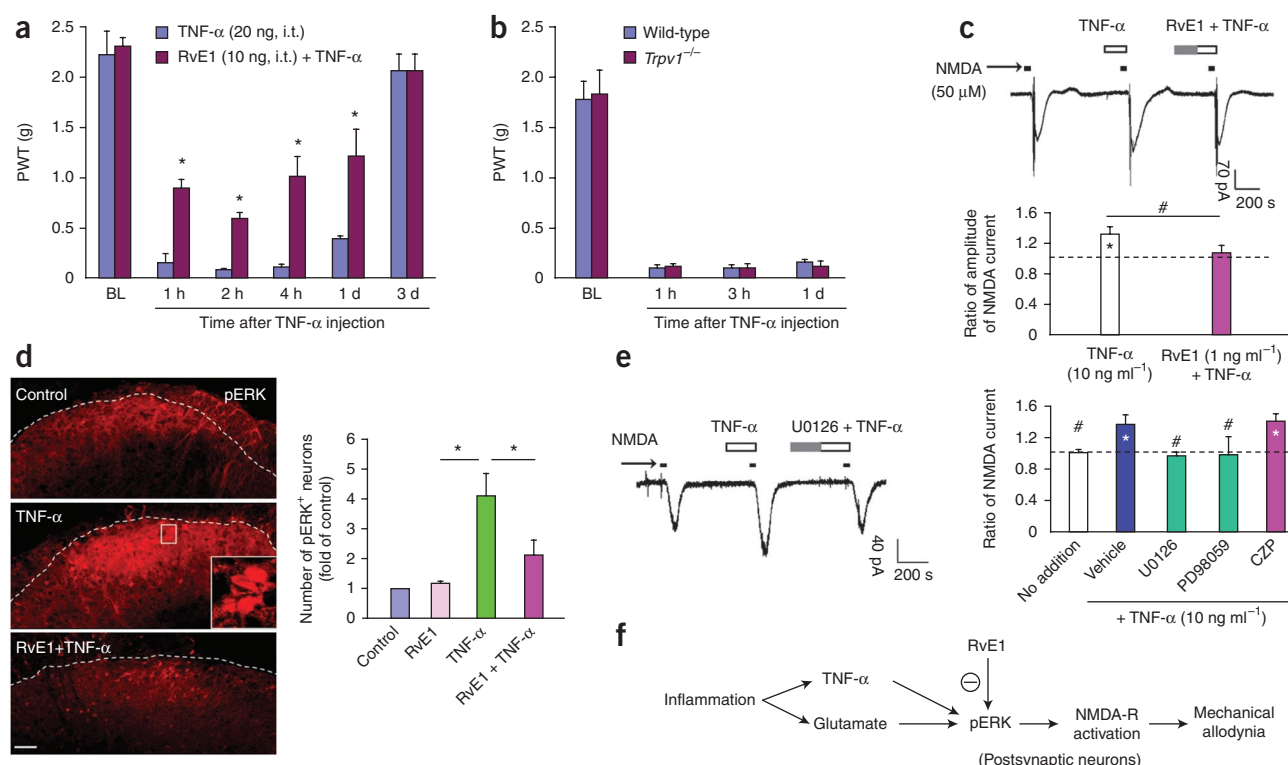


Figure 4 Spinal RvE1 administration attenuates mechanical allodynia and blocks TNF- α signaling in postsynaptic dorsal horn neurons. **(a)** Mechanical allodynia after intrathecal TNF- α and reduction of the allodynia by RvE1 pretreatment. * $P < 0.05$ versus vehicle control, $n = 5$ mice. **(b)** TNF- α -induced mechanical allodynia in wild-type and *Trpv1*^{-/-} mice. $n = 5$ mice. **(c)** NMDA-evoked currents in lamina II neurons by TNF- α and effects of RvE1 on the currents. * $P < 0.05$ versus baseline, # $P < 0.05$, $n = 6$ neurons. **(d)** ERK phosphorylation in superficial dorsal horn neurons of spinal cord slices after perfusion of TNF- α and effects of RvE1 on the phosphorylation. White line, border of the dorsal horn gray matter. * $P < 0.05$, $n = 4$ slices from separate mice. Scale bar, 50 μ m. **(e)** Effects of the MEK inhibitors PD98059 (1 μ M) and U0126 (1 μ M) and capsazepine (CZP, 10 μ M) on NMDA-evoked currents after TNF- α stimulation. * $P < 0.05$ versus corresponding baseline; # $P < 0.05$, versus TNF- α ; $n = 5$ –10 neurons. **(f)** Schematic of RvE1-induced inhibition of inflammatory pain (mechanical allodynia) via postsynaptic mechanisms.

synaptic plasticity in dorsal horn neurons. RvE1 not only abolished TRPV1-induced increases in EPSC frequency and spontaneous pain but also blocked TNF- α -induced increases in EPSC frequency and NMDAR hyperactivity. Activation of the GPCR ChemR23 and inactivation of the ERK signaling pathway in both presynaptic and postsynaptic neurons are required for the antinociceptive effects of RvE1.

Current treatments for inflammatory pain are limited by their side effects, for example, respiratory depression, sedation, nausea, vomiting, constipation, dependence, tolerance and addiction after opioid treatment^{32,33} and serious cardiovascular effects associated with long-term use of COX-2 inhibitors^{1,34}. Additionally, COX-2 inhibitors and local anesthetics impair the resolution of acute inflammation^{4,6}. Although enthusiasm for the use of TRPV1 antagonists is high, these drugs can cause hyperthermia³⁵ and have limited effects on mechanical allodynia. Our results show that resolvins attenuate inflammatory pain without changing basal pain sensitivity. Given the antihyperalgesic efficacy of resolvins and the safety associated with endogenous mediators, resolvins and their metabolically stable analogs may represent a new family of analgesics useful in treating inflammation-associated pain states such as arthritic and post-operative pain. This new analgesic function adds to the beneficial anti-inflammatory and proresolving effects of resolvins².

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

R.-R.J. and C.N.S. formulated the hypotheses, designed and supervised the project and prepared the manuscript; R.-R.J. designed most experiments; Z.-Z.X., T.L. and J.Y.P. conducted behavioral studies; L.Z. performed electrophysiological studies; Z.-Z.X. and L.Z. performed immunohistochemistry; Z.-Z.X. performed siRNA-mediated knockdown, cytokine arrays and western blotting; T.B. performed *in situ* hybridization; R.Y. prepared resolvins and their analogs.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturemedicine/>.

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ONLINE METHODS

Mouse and pain models. We used adult CD1 mice (male, 25–32 g) for most experiments, obtained from Charles River Laboratories. We also used double knockout mice lacking both TNFR-1 and TNFR-2 (*Tnfrsf1a*^{-/-}; *Tnfrsf1b*^{-/-}) or TRPV1 (*Trpv1*^{-/-}) and C57BL/6 wild-type control mice from Jackson Laboratories. All mouse procedures were approved by the Harvard Medical Area Standing Committee on Animals. We injected 20 μ l of diluted formalin (5%, Sigma), carrageenan (1%, Sigma) or complete CFA (Sigma) into the plantar surface of the hindpaw to induce acute, persistent or chronic inflammatory pain, respectively. We also induced postoperative pain through hindpaw incision³⁶ and neuropathic pain by ligation of the spinal nerve³⁷.

Resolvin administration. Synthetic RvE1 and RvD1 were obtained from Cayman Chemical and were qualified according to published physical and biological properties²¹. We suspended RvE1 and RvD1 in 1% or 10% ethanol vehicle and investigated acute (<1 h) and persistent (1–6 h) pain. We delivered the reagents (10 μ l) via intrathecal injection made by a spinal cord puncture between the L5 and L6 levels with a 30-gauge needle³⁸. Detailed methodology is described in the **Supplementary Methods**.

Immunohistochemistry. We deeply anesthetized mice with isoflurane and perfused them through the ascending aorta with 4% paraformaldehyde. We removed spinal cord (L4–L5 segment) and DRG (L4, L5) tissues and postfixed the spinal cord overnight and the DRG for 2 h. We cut spinal cord sections (30 μ m, free-floating) and DRG sections (14 μ m) in a cryostat and performed immunofluorescence. We blocked the sections with 2% goat or donkey serum for 1 h at 22 °C, incubated the sections with primary antibodies overnight at 4 °C and then incubated them with Cy3- or FITC- conjugated secondary antibodies (1 in 400, Jackson Immunolabs) for 1 h at 22 °C. For double immunofluorescence, we incubated tissue sections with a mixture of polyclonal and monoclonal primary antibodies followed by a mixture of FITC- and CY3-conjugated secondary antibodies. We also amplified ChemR23 staining with the TSA (Tyramide Signal Amplification, PerkinElmer) system. Detailed methodology is described in the **Supplementary Methods**.

Primary dorsal root ganglion culture. We aseptically removed DRGs from 4-week-old mice and digested them first with collagenase (1.25 mg ml⁻¹) and dispase-II (2.4 U ml⁻¹) at 37 °C for 90 min and then with 0.25% trypsin for 8 min at 37 °C. We mechanically dissociated DRG cells with a flame-polished Pasteur pipette in the presence of 0.05% DNase I and plated these cells onto poly-D-lysine and laminin-coated slide chambers. We grew DRG cells in a neurobasal defined medium (with 2% B27 supplement, Invitrogen) in the presence of nerve growth factor (Roche Bioscience, 50 ng ml⁻¹) for 24 h and replaced the medium with NGF-free medium before stimulation. For immunocytochemistry, we fixed DRG cells with 4% paraformaldehyde for 30 min and incubated the cells with pERK-specific primary antibody (rabbit, Cell Signaling, 1 in 500) overnight.

Spinal cord slice preparation. As previously reported³⁹, we removed a portion of the lumbar spinal cord (L4–L5) from young mice (3–4 week-old) under urethane anesthesia (1.5–2.0 g per kg body weight, intraperitoneally)

and cut transverse spinal cord slices (600 μ m) on a vibrating microslicer. We perfused the slices with Krebs's solution (8 ml min⁻¹) for >2 h before experiments. We stimulated some slices with TNF- α (10 ng ml⁻¹, 5 min), fixed slices with 4% paraformaldehyde for 1 h and processed thin sections (15 μ m) for pERK immunohistochemistry¹³.

Patch clamp recordings in spinal slices. We performed whole-cell patch-clamp recordings in neurons of lamina II in voltage clamp mode³⁹. After establishing the whole-cell configuration, we held neurons at holding potentials of -70 mV for sEPSC recording. We recorded NMDA-induced currents by bath application of NMDA (50 μ M, Sigma) at a holding potential of -50 mV. We amplified membrane currents with an Axopatch 200A amplifier (Axon Instruments) in voltage-clamp mode. We used pCLAMP 6 and Mini Analysis (Synaptosoft) software to store and analyze the data. We regarded those cells showing >5% changes from the baseline levels as responding ones²⁴.

Behavioral analysis. We habituated mice in the testing environment for 2 d and performed behavioral testing in a blinded manner. We assessed formalin-evoked spontaneous inflammatory pain by measuring the time (in seconds) the mice spent on licking and flinching the affected paws every 5 min for 45 min. We also observed capsaicin-induced spontaneous pain for 10 min. For testing mechanical sensitivity, we put mice in boxes on an elevated metal mesh floor and stimulated hindpaws with a series of von Frey hairs with logarithmically increasing stiffness (0.02–2.56 g, Stoelting), presented perpendicular to the plantar surface. We determined the 50% paw withdrawal threshold by Dixon's up-down method⁴⁰. For testing heat sensitivity, we put mice in plastic boxes and tested heat sensitivity with Hargreaves radiant heat apparatus (IITC Life Science). We adjusted the basal paw withdrawal latency to 9–12 s and set a cut-off of 20 s to prevent tissue damage. We calculated the percentage maximal possible antinociceptive effect (% MPE) with the equation %MPE = [(PL - BL2) / (BL1 - BL2)] \times 100, where BL1 represents baseline latency before inflammation, BL2 represents baseline latency after inflammation but before drug injection and PL represents latency after drug injection.

Statistical analyses. We expressed the data as means \pm s.e.m. and compared the differences between groups with Student's *t* test or analysis of variance followed by Newman-Keuls test. The criterion for statistical significance was *P* < 0.05.

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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